Journal of Integrative Agriculture 2016, 15(10): 2326–2335

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RESEARCH ARTICLE

Sperm pretreatment with glutathione improves IVF embryos development through increasing the viability and antioxidative capacity of sex-sorted and unsorted bull semen

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Abstract

The antioxidant of reduced glutathione (GSH) is the most abundant thiol in cells for the maintenance of the intracellular redox balance. The study aimed to assay the effect of sperm treatment with GSH before incubation with oocytes on the development potential of embryos obtained by *in vitro* fertilization (IVF). Also the mitochondrial membrane potential (ΔΨm), plasma membrane integrity (viability), DNA fragmentation, reactive oxygen species (ROS) content, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, methane dicarboxylic aldehyde (MDA) level as indices of lipid peroxidation in sex-sorted and unsorted sperm from three bulls were investigated using flow cytometry and enzyme-labeled instrument individually. The results showed that 2 mmol L^{-1} GSH increased significantly the cleavage rate (86.68% *vs*. 82.78%), 4- to 8-cell rate (82.30% *vs*. 73.43%) and blastocyst rate (43.15% *vs*. 35.24%) of IVF embryos compared with untreated group. Furthermore, addition of GSH increased significantly the ΔΨm and viability, decreased the ratio of DNA fragmentation in sex-sorted or unsorted semen ($P<0.05$), except the sex-sorted semen from bull 019. Similarly, activities of SOD, CAT and GPx were increased significantly. However, the contents of MDA were decreased significantly both in sex-sorted and unsorted semen treated with GSH ($P<0.05$). These results suggest that sperm pretreatment with GSH during IVF can maintain better the viability and fertility of sperm through reducing apoptosis and increasing the antioxidant capacity, which improves the IVF embryos development.

Keywords: GSH, apoptosis, antioxidant enzymes, unsorted semen, sex-sorted semen

1. Introduction

Sex control technology is used in dairy cow breeding to strengthen selection intensity, speed genetic breeding, and improve quality advantages of the herd (Hutchinson *et al.* 2013). Currently, the only available method used commercially to produce sex-sorted sperm is by flow cytometry based on the small difference in chromosome content

Received 22 March, 2016 Accepted 14 May, 2016

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doi: 10.1016/S2095-3119(16)61402-8

between X and Y mammalian sperm (Seidel 2012). The reliable sorting of X and Y sperms and broader application of sorted sperm can enhance the spread of favorable production traits in cattle industries (Presicce *et al.* 2010). However, sex-sorted sperm has limited widespread and efficient applicability due to generally poor fertility (Clulow *et al.* 2008), in part due to DNA damage and reduced sperm motility resulting from the sorting process (Garner and Seidel 2008). Furthermore, capacitation-like changes and significant decline in longevity occur in sorted sperm. The adverse effects of sorting on spermatozoa and the low number of sperm per dose contribute to lower conception rates after artificial insemination using sorted sperm compared to those using unsorted sperm (Lucena *et al.* 2014).

It has been proposed that reduced fertilization rates of sex-sorted sperm are caused by reactive oxygen species (ROS) produced during the sorting process, which can negatively impact upon sperm because the cell membrane of sperm contains a high content of unsaturated fatty acids, and is sensitive to ROS (Lucena *et al.* 2014). Factors including exposure to Hoechst 33342 stain, high sample dilution, physical stress from high pressures, sudden changes in medium, exposure to intense UV light, and subsequent centrifugation to concentrate the sperm can exacerbate the effects of ROS on sperm (Bathgate 2008). Cryopreservation of sorted sperm can also cause stress to the sperm cells. Manipulation of sperm that can increase lipid peroxidation (LPO) include cooling, long term liquid preservation and freezing; these stresses can cause damage to the structure and physiology of sperm during the cryopreservation process (Watson 2000). The most prominent adverse effects of the cryopreservation process on sperm are loss of motility, reduced viability, and changes in acrosome status and sperm morphology (Guthrie and Welch 2012). The delicate balance of ROS generation and scavenging in sperm is disrupted during the sorting and freezing processes of sex-sorted semen. Excessive ROS impacts the fertilizing ability of sperm, most likely as a result of the multiple roles of ROS in spermatozoa, including sperm motility, morphology, mitochondrial membrane potential (ΔΨm), DNA integrity, and plasma membrane structure (Kim *et al.* 2010).

Supplementation of various antioxidants in the extender, during the sorting and cryopreservation of mammalian sperm has been reported. Chemicals, such as reduced glutathione (GSH), urate, vitamin E, and ascorbic acid, can improve sperm motility, endogenous antioxidant activities, fertilizing ability, and sperm membrane integrity in various species (Alvarez and Storey 1989; Radic *et al.* 2010; Sheikh-Ali *et al.* 2010; Buyukleblebici *et al.* 2014; Sariozkan *et al.* 2014). Glutathione is a tripeptide (gamma-glutamylcysteinylglycine) that is widely distributed in animal somatic cells and gametes. The oxidized (GSSG) and reduced (GSH) glutathione are two intracellular types. Generally, GSH can be converted to GSSG in the presence of glutathione peroxidase (GPx), which is a selenium-containing enzyme. This enzyme also catalyzes the reduction of H_2O_2, which can be reversed by glutathione reductase (Luberda 2005). Notably, a reduction of GSH content in sperm of domestic animals is induced by the freezing procedures (Yeste *et al.* 2014); thus the recovery and increase of intercellular GSH in sex-sorted sperm is important to facilitate normal sperm function for insemination oocytes. GSH has been added in the medium during *in vitro* maturation (IVM) of oocytes (Ishizuka *et al.* 2013), *in vitro* fertilization (IVF), and *in vitro* cultures (IVC) of embryos (Li *et al.* 2014; Sun *et al.* 2015), and can improve developmental competence of fertilized embryos. In recent years, numerous studies have confirmed that GSH, as antioxidant in the semen extender facilitates successful cryopreservation of unsorted sperm from bulls (Sariozkan *et al.* 2009), boars (Estrada *et al.* 2014; Yeste *et al.* 2014), and humans (Gadea *et al.* 2011), and improves the post-thaw motility, viability, membrane integrity, and fertility of spermatozoa.

Nevertheless, the effects of exogenous GSH on apoptosis and antioxidase activity of sex-sorted sperm have not been elucidated clearly. Therefore, this study aimed to investigate the ΔΨm, plasma membrane integrity, DNA fragmentation, ROS content, superoxide dismutase (SOD), catalase (CAT) and Gpx activities, methane dicarboxylic aldehyde (MDA) level in sex-sorted and unsorted sperm after GSH treatment before incubation with oocytes during bovine IVF. The development of bovine IVF embryos was also observed.

2. Materials and methods

2.1. Ethics statement

Experiments were performed according to the Regulations for Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) and approved by the Institutional Animal Care and Use Committee of Chinese Academy of Agricultural Sciences, Beijing, China.

All reagents used in the study were purchased from Sigma-Aldrich (USA) unless otherwise indicated.

2.2. Animals and semen collection

Three bulls (Holstein) aged between 15–18 months were used in the experiment and raised in Shandong Dairy Cattle Center (Jinan, China). They were allowed access to feed

and water *ad libitum* under normal conditions. Semen samples were collected from three bulls using artificial vagina and a total of 24 ejaculates were collected over four weeks.

2.3. Semen sorting and freezing of three bulls

Semen samples from each bull were divided into two parts for sorting or not. For sorting, the semen was diluted in Tyrode's albumin-lactate-pyruvate (TALP) buffer containing 125 mmol L^{-1} Hoechst 33342 for 45 min at 34°C. Then the semen was diluted to 1×10^8 spermatozoa mL^{-1} with TALP containing 4% egg yolk and 0.002% food coloring dye. Stained spermatozoa were sorted for the X-bearing by Moflo XDP system. Sorted sperm were collected in a 50-mL centrifuge tube with Tris fluid containing 20% egg yolk and cooled to 5°C for 90 min. Samples were diluted with an equal volume of Tris extender added with 12% glycerol and centrifuged at 850×g for 20 min at 4°C. After that, the sperm pellet was diluted and the final concentration was adjusted to 1×10^7 spermatozoa mL^{-1}.

The unsorted semen was collected from the same ejaculate as the sorted semen. The sorted and unsorted samples were packaged into 0.5 mL straws with 1×10^7 and 1×10^8 spermatozoa mL^{-1} respectively. Then they were frozen using a routine procedure and preserved in liquid nitrogen.

2.4. Production of bovine embryos by IVF

The IVF procedure was performed according to the method described by Nedambale *et al.* (2006), with minor modifications. One straw of sex-sorted semen from bull 037 was thawed at 38°C for 1 min, resuspended in sperm wash medium (m-BO medium with 10 μg mL^{-1} heparin, 10 mmol L^{-1} caffeine, and 4 mg mL^{-1} BSA), and centrifuged at 500×g for 8 min; wash steps were performed twice. Washed sperm pellets were diluted in sperm fertilization medium (m-BO medium with 4 mg mL^{-1} BSA, 10 μg mL^{-1} heparin) containing 0, 1, 2, 3, 4 mmol L^{-1} GSH, respectively and allowed to acclimate for 3 h. Then sperm pellets were resuspended with fertilization medium after centrifugation at 500×g for 5 min. The concentration of sperm was adjusted into 1×10^6 cells mL^{-1} with fertilization medium.

Ovaries were obtained from mature cows at a local abattoir, washed three times with preheated 0.9% NaCl and transported to the laboratory at 37°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) and matured by culture in IVM medium (Sun *et al.* 2015) at 38.5°C in an atmosphere containing 5% CO_2 and 100% humidity. Following 22–24 h IVM, bovine COCs were washed three times in fertilization medium without GSH. The COCs were incubated with sperm for 8 h in fertilization medium at 38.5°C in an atmosphere containing 5% CO_2 and 100% humidity.

At 18–20 h after IVF, the presumptive zygotes were washed three times and cultured *in vitro* in modified CR1aa medium (Sun *et al.* 2015) supplemented with 6 mg mL^{-1} of BSA for the first 48 h or 10% fetal bovine serum (Gibco, Life Technologies, USA) for the 48–168 h. Cleavage, the numbers of 4- to 8-cell embryos were recorded 48 h after insemination, and blastocyst development was recorded on day 7 after insemination.

2.5. Semen sample preparation for analysis of flow cytometry

One straw of unsorted frozen semen and one straw of sex-sorted semen from each bull were thawed at 38°C for 1 min. The preparation of semen for flow cytometry was the same as the production of bovine IVF embryos. After the treatment of 2 mmol L^{-1} GSH for 3 h, the sperm was washed twice by centrifugation at 500×g for 5 min in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Grand Island, NY, USA). The sperm precipitation was resuspended and concentration was adjusted into 1×10^6 cells mL^{-1}. Every experiment was repeated three times for sorted or unsorted semen from each bull.

2.6. Staining for evaluation of ΔΨm

Sperm ΔΨm was assessed with JC-1 using a staining protocol modified from Garner *et al.* (1997). The lipophilic cation JC-1 was used to differentially label sperm mitochondria with low and high membrane potentials. According to the manufacturer, JC-1 forms multimeric aggregates that, when excited at 488 nm, emit at a wavelength of 590 nm; a high level of fluorescence at 590 nm indicates high mitochondrial potential. JC-1 forms monomers which emit at a range of 525–530 nm when excited at 488 nm; the presence of monomers indicates low membrane potential. JC-1 dye was suspended in DMSO and divided into equal aliquots after filtering and stored at –20°C. JC-1 (5 μg mL^{-1}) was added to each straw and incubated at 37°C in the dark for 15 min. All samples were assessed by flow cytometry. In this way, two sperm subpopulations were identified: events in region P6 (upper left) represented spermatozoa with high ΔΨm (orange fluorescence, FL2), events in region P7 (lower right) represented spermatozoa with low ΔΨm (green fluorescence, FL1).

2.7. Staining for evaluation of sperm plasma membrane integrity

Sperm plasma membrane integrity was assessed using SYBR-14 and propidium iodide (PI), according to manu-

facturer's instructions. This vital stain contains two nucleic acid dyes: SYBR-14 stains spermatozoa with intact plasma membranes green, while PI stains nonviable spermatozoa, causing them to fluoresce red. Each straw of sorted or unsorted sperm was treated with 3 μL SYBR-14 (12 μmol L^{-1}) and 3 μL PI (3 mmol L^{-1}) and was incubated at 37°C in the dark for 15 min. All samples were assessed by flow cytometry. SYBR-14 fluorescence (intact plasma membrane) was detected on detector FL1, PI fluorescence (damaged plasma membrane) was detected on detector FL2.

2.8. Staining for evaluation of ROS production

The current study used 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescent dye to estimate the intracellular ROS level with flow cytometry. The ROS level in fresh spermatozoa and frozen-thawed spermatozoa was estimated as per the procedure described by Mahfouz *et al.* (2010). DCFH-DA (5 μmol L^{-1}) was added to each straw; straws were incubated at 37°C in the dark for 15 min. Labeled spermatozoa were washed with PBS. All samples were assessed by flow cytometry. DCFH-DA fluorescence was detected on detector FL1.

2.9. Staining for evaluation of sperm DNA fragmentation

Sperm DNA fragmentation was evaluated using the *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. A 100 μL aliquot of sperm suspension (1×10^6 cells mL^{-1}) was fixed in paraformaldehyde (4%) on a shaker for 1 h at room temperature and washed with DPBS. The fixative was removed for permeabilization and the cells were resuspended in 0.1% Triton X-100 on ice for 2 min. After washing with PBS, the cell suspension was incubated in 50 μL of labeling solution (containing the TdT enzyme and the label solution fluorescein-dUTP) for 1 h at 37°C in the dark. Negative controls were prepared by incubating slides with labeling solution from the reaction mixture. Fixed and permeabilized sperm with an additional treatment with 2 IU of DNase (recombinant DNase I, Roche Diagnostics) were used as a positive control. The labeled spermatozoa were washed with PBS. All samples were assessed by flow cytometry. TUNEL fluorescence was detected on detector FL1.

2.10. Measurement of antioxidant enzymes activity

Spermatozoa of each group were collected and suspended in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Bull semen samples were centrifuged at 1 000×g for 15 min and supernatant was collected for measurement. The activities of SOD, CAT, GPx and MDA level were determined with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using an enzyme-labeled instrument (Thermo Fisher Scientific, MA, USA).

SOD activity was measured according to the protocol of Panckenko *et al.* (1975), which involves the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at pH 8.0. A single unit of enzyme is defined as the quantity of SOD required to produce 50% inhibition of photochemical reduction of NBT. The absorbance was read at 550 nm against a blank. The SOD activities were demonstrated as U mL^{-1} proteins.

CAT activity was assayed according to the method of Goth (1991). This assay was based on the measurements of hydrogen peroxide (H_2O_2), remaining after the action of CAT. The colorimetric method used a substituted phenol, which coupled oxidatively 4-aminoantipyrine in the presence of H_2O_2 and horseradish. The quinoneimine dye coupling product, which correlated with the amount of H_2O_2 remaining in the reaction mixture, was measured at 405 nm. One unit of CAT decomposed 1 μmol L^{-1} H_2O_2 to oxygen and water per minute at 25°C at a substrate concentration of 50 mmol L^{-1} H_2O_2 (pH 7.0). CAT activity was calculated from the change in absorbance. The activities of CAT were demonstrated with U mL^{-1} proteins.

GPx activity was determined according to the procedures presented previously (Paglia and Valentine 1967). In this assay, GPx catalysed the oxidation of GSH by cumene hydroperoxide. In the presence of GR and NADPH, GSSG was converted to GSH with a concomitant oxidation of NADPH to $NADP^+$. The decrease in the absorbance was measured at an absorbance of 412 nm at 37°C (pH 7.2). The activities were expressed as U L^{-1} proteins.

2.11. LPO determination

The concentrations of MDA, as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction and according to the method described by Yoshioka *et al.* (1979). 4 mL of a reaction mixture containing 3 mL of 0.5% TBA and 1 mL of extract were boiled for 10 min and then quickly cooled to room temperature. The contents of TBA reactive products (MDA) were expressed as nmol mL^{-1}.

2.12. Statistical analysis

All experiments were repeated at least three times. Data in each experiment are expressed as the means±standard deviations (SD). Statistical analyses were performed with

one-way analysis of variance (ANOVA) with Duncan's test for post-hoc analysis using SAS ver. 8 (SAS Institute Inc., Cary, NC, USA). A *P* value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of sperm treated with GSH on the development of bovine IVF embryos

In the present study, a total of 748 embryos (from five independent replicates) were employed to estimate the effect of sex sorted sperm treated with different concentrations of GSH on the development of bovine IVF embryos cultured *in vitro* (Table 1, Figs. 1–2). The results showed that significantly fewer embryos in 4 mmol L^{-1} GSH group reached the cleavage (72%), 4- to 8-cell stage (58%), and blastocyst (29%) compared to the other groups (P<0.05). However, the embryos in 2 mmol L^{-1} GSH group had higher cleavage rate (87%), 4- to 8-cell rate (82%) and blastocyst rate (43%) than that in other groups (P<0.05). There was no significant difference in developmental competence among the 1 mmol L^{-1}, 3 mmol L^{-1} GSH group and control group (P>0.05).

3.2. Supplementation with GSH changes the mean semen-apoptosis parameters of bull spermatozoa

The influence of GSH on the standard semen parameters of unsorted semen and sex-sorted semen was evaluated in three independent experiments (Table 2, Figs. 3–5). For unsorted semen, the addition of GSH resulted in significantly greater viability than that from control groups (P<0.05). Among them, the viability of semen from bull 019 was increased 17.53% and its magnitude was the greatest one. Also the levels of ΔΨm in unsorted spermatozoa treated with GSH were significantly higher than control groups (P<0.05). On the contrary, unsorted semen with GSH treatment had lower DNA fragmentation compared to control groups (P<0.05). Similarly, GSH treatment of sex-sorted semen increased significantly the viability and high ΔΨm compared with untreated groups for three bulls (P<0.05). There were higher DNA fragmentation in sorted semen without GSH treatment from bull 037 and bull 038 than control groups (P<0.05). Generally, samples that were treated with GSH had higher proportion of spermatozoa with viability and high ΔΨm than untreated sperm, while lower percentage of DNA fragmentation sperm in treated sperm than the untreated.

Table 1 Effect of sperm treated with glutathione on the development of bovine *in vitro* fertilized embryos

Groups	No. of inseminated oocytes	Stage of development (%)		
		Cleavage	4- to 8-cells	Blastocyst
Control	148	82.78±3.15 b	73.43±3.30 b	35.24±11.56 b
1 mmol L^{-1} GSH	151	83.89±1.36 ab	74.72±5.92 b	35.13±5.13 b
2 mmol L^{-1} GSH	153	86.67±2.98 a	82.30±2.54 a	43.15±3.87 a
3 mmol L^{-1} GSH	150	80.56±1.36 b	71.28±3.12 b	33.56±4.14 b
4 mmol L^{-1} GSH	146	72.22±3.44 c	58.46±11.16 c	29.29±13.79 c

Data are means±SD. Values with different letters within the same column differ significantly (P<0.05). The same as below,

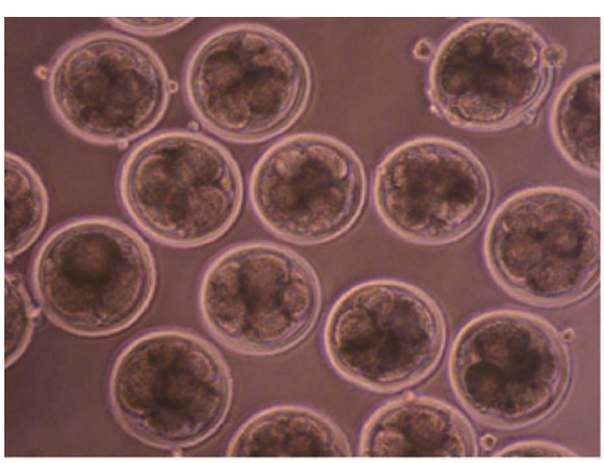

Fig. 1 Bovine embryos at 4- to 8-cell stage obtained by *in vitro* fertilization (IVF) (100×). The oocytes, matured *in vitro*, were fertilized with the sex-sorted sperm from Holstein bull. Embryos were developed to 4- to 8-cell stage during *in vitro* culture.

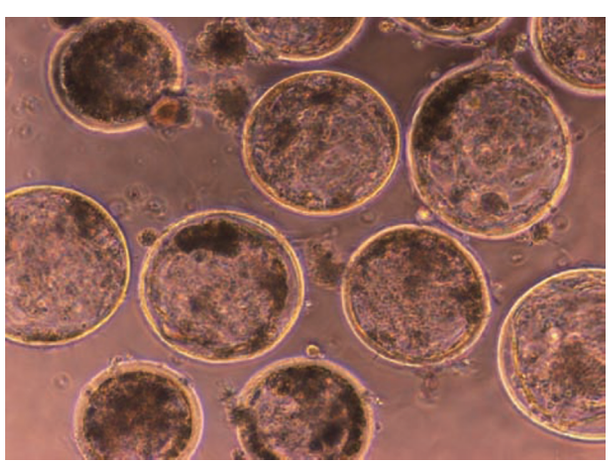

Fig. 2 Bovine blastocysts obtained by IVF (100×). The oocytes, matured *in vitro*, were fertilized with the sex-sorted sperm from Holstein bull. Embryos were developed to blastocysts cultured *in vitro* culture.

Table 2 Sperm quality measures of unsorted and sex-sorted sperm from three bulls

Bull no.	Fertilization medium[1)]	High ΔΨm sperm (%)	DNA fragmentation sperm (%)	Viability (%)
Unsorted				
019	Untreated	23.57±0.74 c	12.87±0.35 a	33.57±1.31 d
	GSH	35.97±0.55 b	11.23±0.56 b	51.10±1.60 a
037	Untreated	25.03±1.46 c	11.90±0.60 ab	40.10±2.25 c
	GSH	44.67±1.54 a	8.70±1.40 cd	45.00±2.43 b
038	Untreated	25.63±4.50 c	9.87±0.15 c	32.73±1.29 d
	GSH	37.40±2.33 b	7.97±0.76 d	39.60±1.60 c
Sex-sorted				
019x	Untreated	6.07±0.40 d	18.40±1.04 b	18.50±1.00 c
	GSH	19.07±0.29 a	17.53±1.36 b	28.20±1.71 a
037x	Untreated	7.80±0.20 c	21.90±3.03 a	17.20±0.56 c
	GSH	14.73±0.70 b	14.90±0.44 c	22.77±0.40 b
038x	Untreated	7.03±0.35 c	19.53±0.21 ab	6.10±0.20 d
	GSH	18.50±0.61 a	10.60±0.60 d	17.60±0.36 c

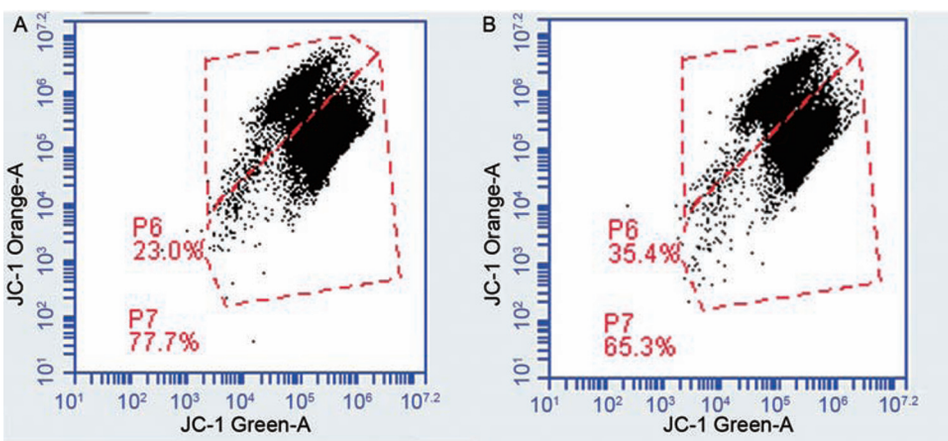

Fig. 3 Mitochondrial membrane potential (ΔΨm) analysis of sperm treated with or without reduced glutathione (GSH). ΔΨm of bull spermatozoa that were untreated or treated with GSH were analyzed using flow cytometry (A, B, respectively). Differences in the ΔΨm were detected. Events in region P6 represent spermatozoa with high ΔΨm; events in region P7 represent spermatozoa with low ΔΨm.

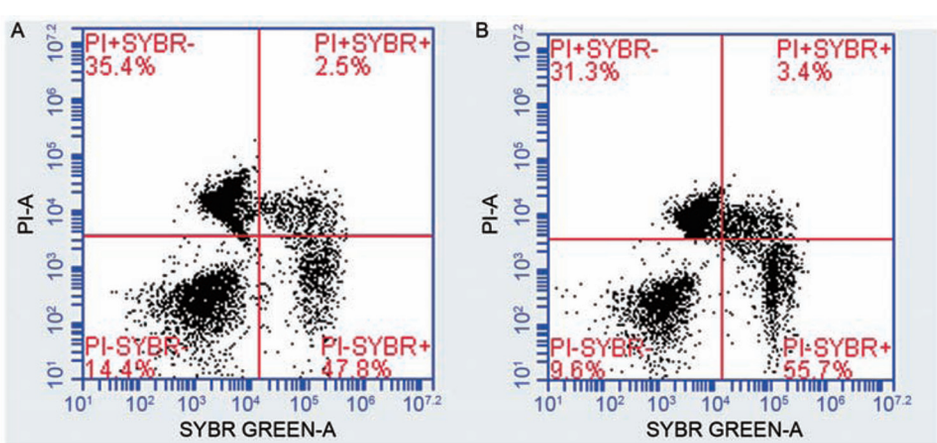

Fig. 4 Viability analysis of sperm treated with or without GSH. Viable spermatozoa were detected using a double-viability stain with SYBR-14 and propidium iodide (PI). Samples of untreated sperm (A) and GSH-treated sperm (B) were stained with SYBR-14 and PI. Within the sorted population there are cellular debris(PI-/SYBR-); viable spermatozoa (PI-/SYBR+); dead spermatozoa (PI+/SYBR-); and dying spermatozoa (PI+/SYBR+).

3.3. Supplementation with GSH changes the oxidative stress parameters

The effects of GSH on antioxidant activities, ROS levels and LPO in unsorted and sex-sorted semen were described in Table 3 and Fig. 6. The addition of GSH to unsorted semen from three bulls resulted in lower ROS and MDA levels than in control groups (P<0.05). Unsorted semen from three bulls treated with GSH had significantly higher SOD, CAT and GPx activities than untreated groups (P<0.05). For sex-sorted semen, the trend of significant difference was same to the unsorted semen from three bulls. That is, the activities of antioxidant enzyme in sperm treated with GSH were higher than that in untreated sperm, while levels of

MDA in treated sperm were lower than the untreated.

4. Discussion

In this experiment, the development of bovine IVF embryos obtained from sorted sperm pretreated with GSH was evaluated. Deeply, the levels of ΔΨm, viability, ROS level, DNA fragmentation, antioxidant enzymes activity, and MDA content were detected after the sex-sorted and unsorted semen from three bulls were pretreated with GSH. The results presented here indicate that the positive effects of GSH improved the embryo development and sperm performance in terms of apoptosis and antioxidation. In this way, 2 mmol L^{-1} GSH counteracts partially injuries resulted from the sorting process and cryopreservation in sex-sorted and unsorted semen. For the antioxidants used in the present study, the findings obtained agreed with the results of Yeste *et al.* (2014), who reported a significant improvement in sperm cryotolerance.

Mitochondria are located in the sperm midpiece and provide energy to the tail filaments (Butts *et al.* 2010). Change in ΔΨm is an efficient indicator of early apoptosis in sperm. The integrity of DNA is vital to the function of spermatozoa. Apoptosis in sperm is morphologically characterized by cellular changes including the appearance of cytoplasmic apoptotic bodies and DNA fragmentation. Our results clearly establish that supplementing fertilization medium with GSH has a positive effect on sex-sorted and unsorted bull semen, as demonstrated by the results of our analysis of semen-apoptosis parameters, oxidative stress parameters, and percentages of cleavage, 4- to 8-cell embryos, blastocyst. These findings are very important, because sperm sorting processes and cryopreservation have been regarded as being very harmful to sperm fertility, and greater utilization of the technique has been hampered because of the resulting lower counts of viable sperm and shorter longevity when compared with the untreated semen (Hollinshead *et al.* 2003). We report that the addition of 2 mmol L^{-1} GSH to the fertilization medium significantly increased sperm viability, also significantly increased ΔΨm, and decreased DNA fragmentation. Our data may lead to the further development of bull sperm sorting or freeze-thawing techniques that

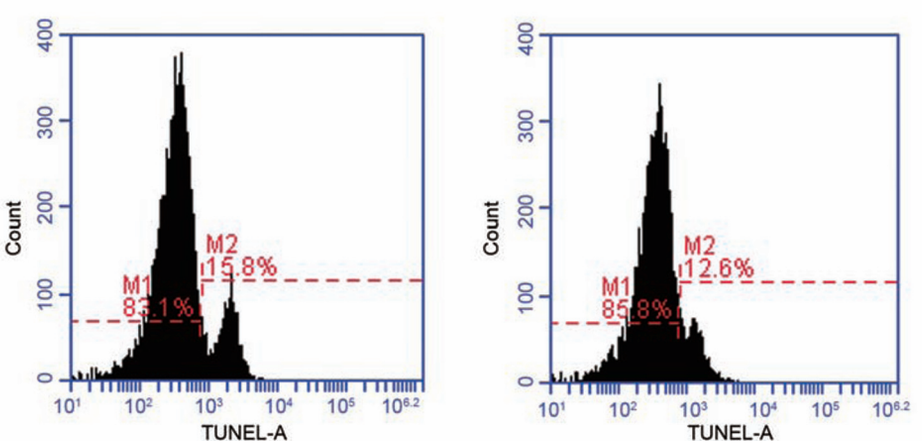

Fig. 5 DNA fragmentation analysis of sperm treated with or without GSH. Spermatozoa from untreated sperm (A) and GSH-treated (B) for evaluation of DNA fragmentation by *In Situ* Cell Death Detection Kit. Events in region M2 represent spermatozoa with DNA fragmentation; events in region M1 represent spermatozoa with normal DNA.

Table 3 Sperm enzyme activity measures of unsorted and sex-sorted sperm from three bulls[1)]

Bull no.	Fertilization medium	ROS level (%)	SOD (U mL^{-1} protein)	CAT (U mL^{-1} protein)	GPx (U L^{-1})	MDA (nmol mL^{-1})
Unsorted						
019	Untreated	47.53±2.49 a	1.56±0.08 c	0.96±0.16 c	194.06±1.02 e	2.09±0.01 b
	GSH	16.23±2.30 d	3.37±0.25 b	1.64±0.12 b	242.32±2.69 a	1.06±0.00 e
037	Untreated	33.27±1.27 b	1.47±0.03 c	1.14±0.11 c	193.54±0.30 e	2.18±0.01 a
	GSH	25.77±2.54 c	3.32±0.07 b	2.00±0.07 a	228.53±1.61 b	1.23±0.00 d
038	Untreated	37.07±5.98 b	1.21±0.05 d	1.07±0.14 c	202.06±4.00 d	2.02±0.00 c
	GSH	32.73±6.42 bc	4.59±0.08 a	1.63±0.05 b	218.03±1.96 c	0.96±0.00 f
Sex-sorted						
019x	Untreated	31.93±0.85 d	1.10±0.09 b	0.50±0.14 b	198.89±2.08 c	2.99±0.01 b
	GSH	17.70±0.96 e	1.53±0.03 a	1.03±0.09 a	222.05±1.00 a	1.90±0.00 d
037x	Untreated	52.70±4.82 b	0.80±0.04 c	0.37±0.18 b	193.61±0.23 d	2.97±0.01 c
	GSH	18.23±0.25 e	1.19±0.01 b	1.15±0.13 a	219.99±1.02 a	1.61±0.00 f
038x	Untreated	59.83±0.49 a	0.83±0.05 c	0.50±0.29 b	203.95±2.09 b	3.16±0.01 a
	GSH	39.60±7.37 c	1.53±0.03 a	1.03±0.06 a	222.20±2.53 a	1.83±0.01 e

[1)] ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, methane dicarboxylic aldehyde .

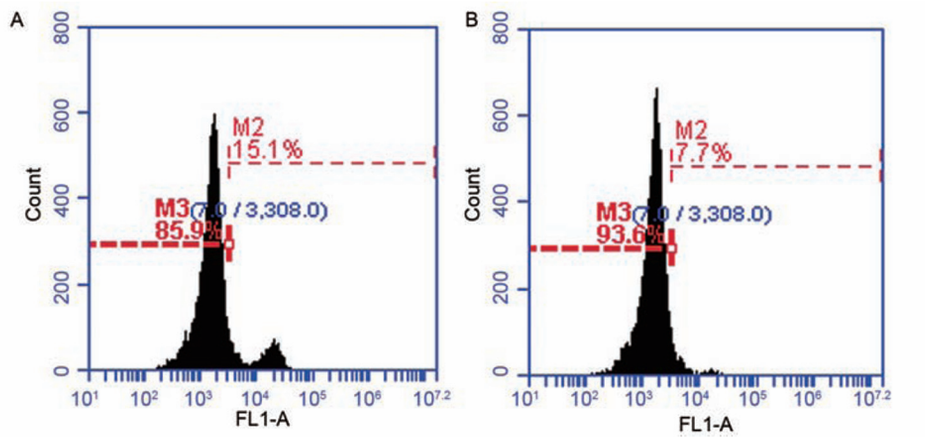

Fig. 6 Reactive oxygen species (ROS) levels of sperm treated with or without GSH. The intracellular ROS level of untreated sperm (A) and GSH-treated sperm (B) were evaluated with DCFH-DA. Events in region M2 represent spermatozoa with high ROS level; events in region M3 represent spermatozoa with low ROS level.

could yield IVF results with sex-sorted semen comparable to those obtained with unsorted semen. Another study has demonstrated that the addition of GSH to the thawing extender increases the function of frozen-thawed boar spermatozoa (Gadea *et al.* 2005a), which supported our results. Similarly, other studies have reported that the addition of GSH prevents sperm motility and acrosome reaction after 24 h incubation or enhances motility of spermatozoa when GSH is added during the centrifugation process (Griveau and Lannou 1994; Parinaud *et al.* 1997), and counteracts strongly cryopreservation-induced sperm apoptosis (Yeste *et al.* 2013). On the contrary, another report has shown that addition of 5, 10, 20 mmol L^{-1} GSH to the freezing extender had no significant effect on progressive motility in fresh spermatozoa (Donnelly *et al.* 2000). The different concentration of GSH may cause the reason. Because less is known about the impact of GSH addition to fertilization medium for IVF with sex-sorted and unsorted semen, our finding that 2 mmol L^{-1} GSH can protect sperm against semen-apoptosis is a very valuable result.

ROS are highly reactive with complex molecules, such as DNA and nucleoprotein, and cause very serious dysfunction, including mitochondrial abnormality and enzyme inactivation (Yeste *et al.* 2013). Many biochemical or mechanical factors have been reported to stimulate the production of ROS in spermatozoa (Gazo *et al.* 2015; Perez *et al.* 2015). The present study showed a significant decrease in ROS levels in sperm treated with GSH compared to untreated sperm from the same bull (P<0.05). This result is consistent with previous experiments, which have reported that addition of 5 mmol L^{-1} GSH to freezing or thawing media can significantly reduce intracellular ROS levels in sperm (Gadea *et al.* 2005a, b). The positive effects of GSH supplementation to fertilization medium by decreasing intracellular ROS levels could be related to a reduction in oxidative stress, as freeze-thawing and sorting procedures have been demonstrated to increase ROS level in spermatozoa (Aitken *et al.* 1996; Kim *et al.* 2011). The antioxidant system comprising SOD, CAT, and GPx is a defensive mechanism against LPO in sperm, and is important in maintaining sperm motility and viability (Aitken and Baker 2004; Gadea *et al.* 2004). MDA production is widely used to determine LPO in the various cell types including sperm cells (Sikka 1996). In the present study, addition of GSH to the fertilization medium extender caused a significant improvement in semen antioxidant enzyme activities, and was similar to results reported in other studies (Gadea *et al.* 2004; Meseguer *et al.* 2007). It has been suggested that addition of GSH prevents MDA production, and resulting in higher SOD, CAT, and GPx activities, compared to the untreated groups for the same bull. A significantly higher MDA production in bull sperm during LPO led to a lower enzyme activity. In agreement with the findings of Sikka *et al.* (1995), our results suggested that the protective effects of GSH on spermatozoa were associated with a reduction in LPO as a consequence of decreasing ROS levels and increasing antioxidant enzyme activities. The SOD, CAT, and GPx enzymes can regulate oxidative stress in sperm by converting peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$) into H_2O and O_2 (Alvarez *et al.* 1987; Bilodeau *et al.* 2001). SOD and CAT eliminate $O_2^{\cdot-}$ produced by NADPH oxidase, and GPx excludes peroxylradicals from various peroxides. Our results indicate that the antioxidant capacity of exogenous GSH could prevent membrane LPO during the fertilization process. Several researchers have reported that treatment of sperm, including sorting processes (Palma *et al.* 2008) and sperm cryopreservation (Watson 2000), may lead to significant increases in measures of sperm quality. This may contribute to the differences obtained between our study and those reported elsewhere.

5. Conclusion

Here we demonstrate the effectiveness of supplementation of GSH in the extender of fertilization medium to improve the performance of sex-sorted and unsorted semen. The

use of additives may be recommended to improve and facilitate semen cryopreservation systems in the bovine breeding industry.

Acknowledgements

This work was partially supported by the grants from the National Science and Technology Support Program of China (2012BAD12B01), the Basic Research Fund of Institute of Animal Science, Chinese Academy of Agricultural Sciences (2013ywf-zd-2), the Agricultural Science and Technology Innovation Program, China (ASTIP-IAS06), and the China Agriculture Research System (CARS-37). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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(Managing editor ZHANG Juan)